Accepted Manuscript

This is an Accepted Manuscript of the following article:

Erika Castrignanò, Zhugen Yang, Richard Bade, Jose A. Baz-Lomba, Sara Castiglioni, Ana Causanilles, Adrian Covaci, Emma Gracia-Lor, Felix Hernandez, Juliet Kinyua, Ann-Kathrin McCall, Alexander L.N. van Nuijs, Christoph Ort, Benedek G. Plósz, Pedram Ramin, Nikolaos I. Rousis, Yeonsuk Ryu, Kevin V. Thomas, Pim de Voogt, Ettore Zuccato, Barbara Kasprzyk-Hordern. Enantiomeric profiling of chiral illicit drugs in a pan-European study. Water Research. Volume 130, 2018, pages 151-160, ISSN 0043-1354.

The article has been published in final form by Elsevier at

http://dx.doi.org/10.1016/j.watres.2017.11.051

It is recommended to use the published version for citation.

Enantiomeric profiling of chiral illicit drugs in a pan-European study

Erika Castrignanò[a**], Zhugen Yang[a,b], Richard Bade[c,d], Jose A. Baz-Lomba[e], Sara Castiglioni[f], Ana Causanilles[g], Adrian Covaci[h], Emma Gracia-Lor[c,f], Felix Hernandez[c], Juliet Kinyua[h], Ann-Kathrin McCall[i], Alexander L. N. van Nuijs[h], Christoph Ort[i], Benedek G. Plósz[j,k], Pedram Ramin[j,l], Nikolaos I. Rousis[f], Yeonsuk Ryu[e], Kevin V. Thomas[e,m], Pim de Voogt[g,n], Ettore Zuccato[f] and Barbara Kasprzyk-Hordern[a*]

[a] Department of Chemistry, Faculty of Science, University of Bath, Bath, BA2 7AY, United Kingdom (UK)

[b] Division of Biomedical Engineering, School of Engineering, University of Glasgow, Oakfield Road, Glasgow G12 8LT, UK

[c] Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat s/n, E-12071, Castellón, Spain

[d] School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, South Australia 5000, Australia

[e] Norwegian Institute for Water Research (NIVA), Gaustadalleen 21, 0349, Oslo, Norway

[f] IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Department of Environmental Health Sciences, Via La Masa 19, 20156, Milan, Italy

[g] KWR Watercycle Research Institute, Chemical Water Quality and Health, P.O. Box 1072, 3430 BB, Nieuwegein, The Netherlands

[h] Toxicological Center, Department of Pharmaceutical Sciences, Campus Drie Eiken, University of Antwerp, Universiteitsplein 1, 2610, Wilrijk-Antwerp, Belgium

[i] Eawag, Swiss Federal Institute of Aquatic Science and Technology, CH-8600, Dübendorf, Switzerland

[j] Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet, Building 115, DK-2800M, Kgs. Lyngby, Denmark

[k] Department of Chemical Engineering, University of Bath, Claverton Down, Bath, BA2 7AY, UK

[l] Process and Systems Engineering Center (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 229, 2800 Kgs. Lyngby, Denmark

[m] Queensland Alliance for Environmental Health Science (QAEHS), University of Queensland, 39 Kessels Road, Coopers Plains, QLD, 4108, Australia

[n] IBED-University of Amsterdam, The Netherlands

* Corresponding author: Barbara Kasprzyk-Hordern, E-mail: b.kasprzyk-hordern@bath.ac.uk

** Corresponding author: Erika Castrignanò, E-mail: E.Castrignano@bath.ac.uk

Abstract

The aim of this paper is to present the first study on spatial and temporal variation in the enantiomeric profile of chiral drugs in eight European cities. Wastewater-based epidemiology (WBE) and enantioselective analysis were combined to evaluate trends in illicit drug use in the context of their consumption vs direct disposal as well as their synthetic production routes. Spatial variations in amphetamine loads were observed with higher use in Northern European cities. Enantioselective analysis showed a general enrichment of amphetamine with the *R*-(-)-enantiomer in wastewater indicating its abuse. High loads of racemic methamphetamine were detected in Oslo (EF = 0.49 ± 0.02). This is in contrast to other European cities where *S*-(+)-methamphetamine was the predominant enantiomer. This indicates different methods of methamphetamine synthesis and/or trafficking routes in Oslo, compared with the other cities tested. An enrichment of MDMA with the *R*-(-)-enantiomer was observed in European wastewaters indicating MDMA consumption rather than disposal of unused drug. MDA's chiral signature indicated its enrichment with the *S*-(+)-enantiomer, which confirms its origin from MDMA metabolism in humans. HMMA was also detected at quantifiable concentrations in wastewater and was found to be a suitable biomarker for MDMA consumption. Mephedrone was only detected in wastewater from the United Kingdom with population-normalised loads up to 47.7 mg 1000 people^{-1} day^{-1}. The enrichment of mephedrone in the *R*-(+)-enantiomer in wastewater suggests stereoselective metabolism in humans, hence consumption, rather than

direct disposal of the drug. The investigation of drug precursors, such as ephedrine, showed that their presence was reasonably ascribed to their medical use.

Keywords

Wastewater-based epidemiology; Illicit Drugs; Chiral Drugs; Enantioselective analysis.

1. Introduction

Since the first study by Zuccato et al. (Zuccato, Chiabrando et al. 2005), where wastewater-based epidemiology (WBE) was introduced as an approach to estimate community-wide illicit drug use trends, WBE has proven to provide valuable and complementary information to traditional epidemiological approaches (Thomas and Reid 2011, Kasprzyk-Hordern, Bijlsma et al. 2014). Indeed, the analysis of carefully selected biomarkers, which are often unique human urinary metabolic excretion products, has allowed for near real-time profiling of the community-wide use of a number of illicit drugs (Thomas, Bijlsma et al. 2012, Ort, van Nuijs et al. 2014), new psychoactive substances (NPS) (Reid, Derry et al. 2014, Castiglioni, Borsotti et al. 2015), alcohol (Reid, Langford et al. 2011) and tobacco (Castiglioni, Senta et al. 2014) use and counterfeit medicines (Causanilles, Emke et al. 2016). The study by Zuccato et al. was followed and further developed by other research groups (Van Nuijs, Pecceu et al. 2009, van Nuijs, Pecceu et al. 2009, Karolak, Nefau et al. 2010, Metcalfe, Tindale et al. 2010, Terzic, Senta et al. 2010, Reid, Langford et al. 2011, van Nuijs, Castiglioni et al. 2011). The first Europe-wide study in 2011, led by the SCORE group (www.score-cost.eu), involved 19 cities and estimated temporal and spatial drugs use trends across Europe (Thomas, Bijlsma et al. 2012). This was followed by Europe-wide monitoring of 23 cities in 2012 (Ort, van Nuijs et al. 2014) and then 42 cities in 2013 (http://www.emcdda.europa.eu/topics/pods/waste-water-analysis 2016). WBE is currently used to report on world-wide illicit drug use trends (Lai, O'Brien et al. 2016, Tscharke, Chen et al. 2016) and feeds into the Europe-wide evidence based early warning system managed by the European Monitoring Centre for Drugs & Drug Addiction (EMCDDA) ~~(http://www.emcdda.europa.eu/activities/wastewater-analysis)~~ (http://www.emcdda.europa.eu/activities/wastewater-analysis).

There are several key stages that need to be considered when developing new WBE applications: (i) biomarker selection; (ii) collection of representative wastewater samples; (iii) measurement of biomarkers in wastewater; (iv) calculation of population-normalised mass loads and, finally, (v) estimation of the consumption *pro capita*. Biomarker selection is considered to be of critical importance. This cannot be limited to the parent drug itself if the determination of drug consumption estimate is the aim, since bias related to disposal of the unused drug might take place. A biomarker should be uniquely formed in the body, be stable and present in wastewater at quantifiable concentrations. Furthermore, the impact of transformation of biomarkers in sewer biofilm/suspended solids between the discharge and the sampling points should be considered as it could affect the detected amount of the analytes, thereby influencing epidemiological observations (McCall, Scheidegger et al. 2016, Ramin, Libonati Brock et al. 2016). Unfortunately, as it is not always possible to select a unique metabolic biomarker, different solutions need to be sought. One of the innovative approaches focuses on enantiomerism of chiral drugs and their stereoselective human metabolism [26].

Enantiomeric profiling can complement WBE data with valuable information on abuse trends and potency of chiral drugs. It can also help with distinguishing between the legal and illicit use of drugs, as well as providing an indication of actual consumption as opposed to disposal of non-consumed drugs [2]. This is because drug synthesis is associated with different chiral signatures that depend on the routes of synthesis. Furthermore, chiral drugs undergo stereoselective disposition in humans leading to changes in their chiral signature (expressed as enantiomeric fraction, EF) (Kasprzyk-Hordern 2010) when excreted.

The potential of enantioselective analysis for WBE purposes has thus far only been demonstrated in a few limited studies focussing on (i) verification of the fate of chiral drugs during wastewater treatment and in the environment (Camacho-Muñoz 2015), (ii) confirmation of origin of amphetamine found in wastewater in the United Kingdom (UK) (Kasprzyk-Hordern and Baker 2012) and (iii) confirmation of MDA present in wastewater as a result of MDMA consumption rather than MDA use (Kasprzyk-Hordern and Baker 2012). Vázquez-Roig et al. (Vazquez-Roig, Kasprzyk-Hordern et al. 2014) reported usage patterns of chiral drugs in the catchment area of Valencia (Spain), by linking selective enrichment of MDMA with the *R*-(-)-enantiomer in wastewater to human consumption. Enantioselective analysis also proved invaluable in establishing that the unexpectedly high quantity of MDMA detected during a monitoring campaign in 2011 in Utrecht was due to direct disposal of unused MDMA as a consequence of a police raid at a nearby illegal production facility (Emke, Evans et al. 2014) and not as a result of high levels of consumption. Similarly, Petrie et al. (Petrie, Youdan et al. 2016) linked high levels of fluoxetine in wastewater with the disposal of the unused drug rather than its consumption. Recently, Castrignanò et al. (Castrignanò, Lubben et al. 2016) found mephedrone enriched with R-(+)-enantiomer in wastewater in the UK suggesting human use.

Despite these findings, a limited number of studies have correlated the enantiomeric composition of chiral biomarkers to official statistics (Camacho-Muñoz 2015). Hence, this is the first pan-European study aimed at investigating enantiomeric profiling of “common” drugs of abuse, NPS and chiral drug precursors in eight cities from different countries with a total population equivalent of 4.9 million. The focus of this research was to:

- quantify selected drugs in wastewater from eight European cities,

- verify if drug residues in wastewater originated from the direct disposal of unused drugs into the sewer system or their consumption.~~;~~

2. Experimental

2.1. Chemicals and materials

The following chiral analytes were selected in this study (Figure S1): (±)-mephedrone, (±)-4-hydroxy-3-methoxymethamphetamine (HMMA), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-4-hydroxy-3-methoxyamphetamine (HMA), (±)-methamphetamine, (±)-amphetamine, (±)-3,4-methylenedioxyamphetamine (MDA), (±)-3,4-methylenedioxy-N-ethyl-amphetamine (MDEA), (±)-ephedrine, (±)-pseudoephedrine, (±)-para-methoxyamphetamine (PMA), (±)-norephedrine. Table S1 shows properties of all analytes. Amphetamine-D_5, methamphetamine-D_5, mephedrone-D_3, MDA-D_5, MDMA-D_5, MDEA-D_5 and *1S,2R*-(+)-ephedrine-D_3 were used as internal standards (ISs).

All standards and ISs were of the highest purity available (>97%). Stock and working solutions of standards were stored at -20 °C. Methanol, acetonitrile and ammonium acetate were purchased from Sigma Aldrich, UK. Ultrapure water was obtained from MilliQ system (UK). Deactivation of the glassware was carried out as described in (Castrignanò, Lubben et al. 2016) to prevent the adsorption of basic analytes to the hydroxyl sites on the glass surface.

2.2. Sample collection, storage and sample preparation

24-hour composite wastewater influent samples were collected over seven consecutive days in March 2015 from wastewater treatment plants (WWTPs) across Europe using best practice sampling protocol (Castiglioni, Thomas et al. 2014). The week in March was chosen as a “routine week”, in which no national and local festivities were taking place. Sampling sites were in Norway (Oslo), United Kingdom (Bristol), Denmark (Copenhagen), The Netherlands (Utrecht), Belgium (Brussels), Switzerland (Zurich), Italy (Milan) and Spain (Castellón). Table S2 provides information on population and flow for the selected cities in the study. After collection, samples were transported to the local laboratory in refrigerated conditions and shipped on ice blocks to the UK within 24 hours. A fully validated analytical method was used for the detection and quantification of chiral drugs of abuse in wastewater as described elsewhere (Castrignanò, Lubben et al. 2016).

2.3. Sample analysis

Samples were analysed in triplicate using enantioselective high performance liquid chromatography coupled with tandem mass spectrometry system. Separation of all chiral analytes was undertaken with a CHIRALPAK® CBH HPLC column 5 µm particle size, L × I.D. 10 cm × 2.0 mm with a chiral-CBH guard column 10 × 2.0 mm, 5 µm particle size (Chiral Technologies, France) using a Waters ACQUITY UPLC® system (Waters, Manchester, UK) under isocratic conditions at a 0.1 mL min^{-1}. The mobile phase was a solution 1 mM ammonium acetate/methanol 85:15 v/v. The temperature was kept at 4 °C in the ACQUITY UPLC™ autosampler, whilst at 25 °C in the column compartment. The injection volume was set at 20 µL.

A triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an electrospray ionisation source was used in positive mode operating in the multiple reaction monitoring (MRM) mode. Table S3 shows MRM transitions used for selected analytes. MassLynx 4.1 (Waters, UK) was used to control the Waters ACQUITY system and the Xevo TQD. Data processing was carried out using TargetLynx software (Waters, Manchester, UK). Method validation data are provided in Tables S4-S8.

2.4. Calculations

Enantiomeric fraction (EF) was calculated using the following equation (1):

$$EF = \frac{(+)}{[(+)+(-)]} \quad (1)$$

where (+) is the concentration of (+)-enantiomer or the first eluted enantiomer and (-) is the concentration of (-)-enantiomer or the second eluted enantiomer. EF equals 0.5 in the case of a racemate, whilst 1 or 0 in the case of the enantiopure compound.

In order to obtain daily mass loads, the concentrations of analytes expressed in ng L^{-1} (see Table S9) were multiplied by the flow rate (L day^{-1}) and then normalised by the population size of the catchment area. This was essential for comparing data coming from different cities involved in the study.

All relevant information on the selected chiral illicit drugs is gathered in Table ~~S9~~S10. It includes: biomarkers used as drug target residue (DTR), urinary excretion data, correction factors (CFs) used for WBE estimates, EF expected in urine after human metabolism (EF_{urine}), EF calculated from illegal synthesis of the drug ($EF_{illegal\_synth}$), information derived from the legal use of the drug with EF derived from the legal use of the drug ($EF_{legal\ source}$) and consumption estimates from official health statistics and from wastewater analysis. CF was calculated as the ratio between the molar ratio of the drug and its DTR and the urinary excretion data.

Estimated community-wide consumptions were calculated using population-normalised mass loads and CF.

3. Results and Discussion

3.1. Amphetamines

Data on amphetamines consumption, reported by the European drug report 2015 (as a sum of amphetamine and methamphetamine), showed that 1.3 million Europeans within the ages of 15 - 34 used amphetamines in the last year (EMCDDA 2015). This data was obtained using the EMCDDA’s five key epidemiological indicators, which consist of “estimates of recreational use (based mainly on surveys), estimates of high-risk use, drug-related deaths, infectious diseases and drug treatment entry” along with Reitox focal points and other sources (EMCDDA 2015). In this work, we applied WBE to estimate amphetamine and methamphetamine use in eight European cities. Unfortunately, no metabolic biomarkers of amphetamine and methamphetamine are validated for a reliable estimation of their abuse via WBE. Therefore, amphetamine and methamphetamine themselves are commonly used as biomarkers. This constitutes a problem since the analysis of parent drugs does not allow for distinguishing between consumed and unconsumed (meth)amphetamine. Additionally, amphetamine is also a metabolite of other (prescription) drugs, such as fenethylline, fenproporex, methamphetamine (Baselt) and selegiline (Ort, van Nuijs et al. 2014). Furthermore, the percentage of the unchanged amphetamine fraction in urine can change due to changes in urine pH (Table ~~S9~~S10), leading to high uncertainty of calculations and possible over or underestimation of amphetamine use. The awareness of this uncertainty is well recognised in the scientific community studying amphetamine use using WBE (Chiaia-Hernandez, Banta-Green et al. 2011), (Kasprzyk-Hordern, Dinsdale et al. 2009), (Postigo, Lopez de Alda et al. 2010), (van Nuijs, Mougel et al. 2011). As reported by Ort et al. (Ort, van Nuijs et al. 2014), the estimation of the amphetamine consumption has to be carried out in the context of methamphetamine data to distinguish between drug consumption from its metabolism. However, verification of the amphetamine/methamphetamine ratio cannot provide comprehensive information on drug consumption against direct disposal of unused drug. Additional evidence is therefore needed to distinguish between amphetamine abuse from its direct disposal or its usage as a prescription drug. The phenomenon of enantiomerism of amphetamines may provide invaluable insight (see section S1-2 for further information).

3.1.1. Amphetamine

Population-normalised amphetamine loads were <5 mg day^{-1} 1000 people^{-1} in Milan to a maximum weekly average value of 122.3 mg day^{-1} 1000 people^{-1} in Oslo, which shows higher amphetamine prevalence in Northern Europe (Figures 1a and S2, estimated consumptions also shown ~~in~~ Table S10S11). There was a decreasing amphetamine usage from Northern to Southern cities with only Italian and Spanish cities notably below the overall mean load of 28 mg day^{-1} 1000 people^{-1} reported in the 2013 European study (Ort, van Nuijs et al. 2014). By looking at the results from previous monitoring studies undertaken since 2012 (Ort, van Nuijs et al. 2014), temporal trends show that amphetamine loads increased in Oslo, Copenhagen, Brussels and Milan, even if they are very low for the latter city. They remained stable in Bristol and decreased in Zurich and in Utrecht.

Enantiomeric profiling revealed that amphetamine in wastewater was enriched with the *R*-(-)-enantiomer in most European cities (EF_{ww}<0.5, EF determined in the wastewater is referred as EF_{ww};) the enrichment was significant as the unpaired t-test showed “t Stat > t Critical one-tail” 8.25 > 1.81 for α = 0.05 and 8.25 > 4.14 for α = 0.001, p one-tail 0.0000045 < 0.001). This could indicate the consumption of racemic amphetamine (see section S1 for further discussion). Interestingly, amphetamine was found to be enriched with *S*-(+)-enantiomer in Milan (EF_{ww}=0.67±0.16). This suggests either usage of *S*-(+)-amphetamine (prescribed or illicit) or its formation as a result of metabolism of methamphetamine. Indeed, the illicit origin of amphetamine is very likely as methamphetamine was also found to be enriched with the S-(+)-enantiomer (see section 3.1.2).

3.1.2. Methamphetamine

In this study, population-normalised methamphetamine loads ~~ranged from <MQL in wastewater from Utrecht and Castellón~~ were <5 mg day^{-1} 1000 people^{-1} in Bristol and Brussels to a maximum value of 172.4 mg day^{-1} 1000 people^{-1} in Oslo wastewater (Figures 1b and S2, estimated consumptions in Table ~~S11~~S12). According to the EMCDDA (EMCDDA 2015), high methamphetamine seizures were reported in Norway. A correlation (not statistically significant) was found between amount seized and loads in wastewater (Baz-Lomba, Salvatore et al. 2016). Zurich wastewater was found to have the second highest methamphetamine loads of 20.2 mg day^{-1} 1000 people^{-1} as a weekly average of eight cities. Estimates in Copenhagen and Brussels were below the overall mean value. Wastewater from other European cities contained low levels. Despite being below the European average (http://www.emcdda.europa.eu/topics/pods/waste-water-analysis 2016), data from Milan has shown that the methamphetamine load has doubled when compared to data from the same area in 2013-14 and reaching 2012 loads.

Enantiomeric profiling of European wastewater revealed that methamphetamine used in most European locations tested was the enantiopure *S*-(+)-methamphetamine with EF_{ww} ranging from 0.89±0.01 to 1.00±0.00. Norwegian wastewaters were an exception as they contained racemic methamphetamine ($EF_{ww(n=7)}$=0.49±0.02), which also indicated direct disposal of unused (±)-methamphetamine. Indeed, it has been reported by the EMCDDA (EMCDDA 2014) that methamphetamine available in Norway (and in Sweden) is mainly produced from phenylacetone and trafficked as racemate from Lithuania (see section S2 for further information). This is because clandestine production facilities in Lithuania tend to utilise a different synthetic route for methamphetamine production than facilities in Central Europe. Interestingly, since *S*-(+)-methamphetamine is the most potent psychotropic enantiomer (Freeman and Alder 2002) of methamphetamine, one can conclude that despite the lower usage of methamphetamine in Zurich, Copenhagen, Brussels and Milan, the potency of the drug is much higher in these cities than in Oslo.

3.2.MDMA and MDA

The European drug report 2015 stated that 1.8 million Europeans with an age range from 15 and 34 used ecstasy (with MDMA as the main ingredient) in the last year, with a low and stable prevalence trend (EMCDDA 2015). Europe-wide MDMA usage was also estimated using WBE (Thomas, Bijlsma et al. 2012, Ort, van Nuijs et al. 2014). Unfortunately, so far estimations are based on quantification of MDMA as a DTR in wastewater. Such an approach does not allow for accurate evaluation of MDMA consumption against the direct disposal of unused drug. There are two possible solutions: (1) specific metabolic biomarkers should be sought as MDMA is known to metabolise to MDA, DHMA and HMMA (Figure S3) (Castrignanò, Lubben et al. 2016, Gonzalez-Marino, Zuccato et al. 2017), and (2) enantiomeric profiling should be implemented as MDMA undergoes stereoselective metabolism leading to the formation of chiral metabolites (see section S3 for further information).

In the current study, population-normalised MDMA loads ranged from a minimum average value of 3.2 mg day^{-1} 1000 people^{-1} in Castellón to a maximum value of 62.0 mg day^{-1} 1000 people^{-1} in Utrecht (Figures 2 and S2, estimated consumptions also in Table ~~S12~~S13). Increasing MDMA loads were found during the weekend in all the countries involved, with the exception of Utrecht that had also high MDMA loads on a weekday. The overall MDMA weekly mean in 2013 was 18 mg day^{-1} 1000 people^{-1} (Ort, van Nuijs et al. 2014). A geographical trend of MDMA loads from North to South was also found. Indeed, Northern European cities (except for Brussels) were mostly above the average. Enantiomeric profiling revealed that MDMA in wastewater is enriched with *R*-(-)-MDMA ($0.32<EF_{ww}<0.40$). This indicates that MDMA retrieved in wastewater comes from consumption, due to the stereoselective metabolism of MDMA in humans. Figure S3 shows expected EF_{ww}s in wastewater for MDMA consumption using the conditions reported in Castrignanò et al (Castrignanò, Lubben et al. 2016). Although illicit MDMA production sites are presumably mainly located in The Netherlands and Belgium (as mentioned in the EMCDDA report (EMCDDA 2015)), MDMA loads in Utrecht and Brussels were linked to human consumption rather than its direct disposal. In contrast, incidental findings in the wastewater of the ~~cities~~ city of Utrecht (Emke, Evans et al. 2014)~~[24] and Eindhoven [5]~~ have shown that aberrantly high loads of (±)-MDMA can occur and can be ascribed to disposal of the unconsumed drug.

The hypothesis that MDMA was present in European wastewaters as a result of its consumption was further evidenced by the study of MDA and its chiral signature. MDA can be a drug of abuse itself or a metabolite of MDMA and MDEA (3,4-methylenedioxyethylamphetamine). It is therefore of utmost importance to verify the origin of MDA. It does not have any medical applications and is available on the illicit market as a racemate (Karch and Drummer 2001) ($EF_{illegal\_synth}$=0.5). This is due to its non-stereoselective synthetic route. Similarly to MDMA, MDA's metabolism favours the *S*-(+)-enantiomer (Meyer, Peters et al. 2009). Therefore, if MDA is consumed, it will be excreted in urine enriched with the *R*-(-)-enantiomer ($EF_{urine}<0.5$). However, if MDA is formed as a result of the metabolism of MDMA or MDEA, it will be present in urine (and in wastewater) enriched with *S*-(+)-enantiomer (Levine 2003, Kasprzyk-Hordern, Kondakal et al. 2010) ($EF_{urine}>0.5$). In this study, MDEA, for which a new CF was proposed, was not detected in any European location. The highest loads of MDA were recorded in Utrecht with 3.2 mg day^{-1} 1000 $people^{-1}$, followed by Bristol with 1.9 mg day^{-1} 1000 $people^{-1}$ and Oslo with 0.5 mg day^{-1} 1000 $people^{-1}$ at average weekly loads (Table ~~S13~~S14). Interestingly, these countries have also high MDMA use, which led us to the conclusion that MDA could be present in wastewater due to consumption of MDMA. In most cases, MDA was found in wastewater enriched with *S*-(+)-enantiomer proving that its presence was associated with the consumption of MDMA, with exception of three days in Bristol, one day in Oslo and in Utrecht when MDA was enriched of the *R*-(-)-form. This could indeed indicate an abuse of MDA. In the case of racemic MDA found in Utrecht for two days, this could indicate a combination of either the consumption of MDA and MDMA (most likely as HMMA data confirmed it) or simply the direct disposal of non-consumed MDA.

As MDA is a minor and not exclusive metabolite of MDMA, other metabolites were also considered as possible DTRs for MDMA consumption: HMA and HMMA. HMA was detected at 3.4 mg day^{-1} 1000 people^{-1} as weekly average in three days of the monitoring week in the Dutch city (Saturday, Sunday and Monday) and at 7.4 mg day^{-1} 1000 people^{-1} in two days in Bristol samples (Sunday and Monday) (Table ~~S14~~S15). Because of the low percentage of excretion of HMA after a dose of MDMA, its choice as MDMA DTR could be considered only in the case of high MDMA intake. Indeed, it was only found in those countries reporting the highest levels of MDMA. EF_{ww} showed values close to 0.5 when high HMA loads were detected. However, the relevance of enantioselective analysis is difficult to comment on because of the low number of positive samples for HMA.

HMMA, on the other hand, was found in wastewater at ng/L level in six cities (i.e. no HMMA was detected in Oslo and Milan) (Table ~~S15~~S16). HMMA's excretion is 20%, which indicates that it could be used as MDMA's DTR. Due to the stereoselective metabolism of MDMA, HMMA and its glucuronide derivative are formed enriched with *S*-(+)-enantiomer. Interestingly, HMMA sulphate is formed via non-stereoselective route (Schwaninger, Meyer et al. 2012). In this study, HMMA was enriched with the second eluting enantiomer. Assuming the same elution order of MDMA enantiomers for HMA and HMMA under the same chromatographic conditions, the second-eluting enantiomer could be assigned as *S*-(+)-enantiomer. The expected EF_{ww} would then be >0.5 for HMMA. Therefore, we hypothesize that, if an enrichment of *R*-(-)-MDMA occurred in the case of consumption, the presence of *S*-(+)-HMMA would be observable along with an EF>0.5. Consumption estimates from wastewater analysis were calculated taking into consideration the following DTRs: MDMA itself (CF applied was 1.5 as it was widely used in literature (Zuccato, Chiabrando et al. 2008, Postigo, Lopez de Alda et al. 2010, Nefau, Karolak et al. 2013) even though a new CF of 6.7 was proposed in this study as a result of the most recent excretion data), MDA, HMMA and HMA (see CF in Table ~~S9~~S10). The estimates obtained with MDA and HMA showed that these compounds were not suitable as biomarkers of MDMA consumption. Indeed, the estimates calculated by using HMMA were quite superimposable to the parent drug MDMA, except for Oslo.

3.3. Mephedrone

Mephedrone was previously detected in the UK (Castrignanò, Lubben et al. 2016), Italy (González-Mariño, Gracia-Lor et al. 2016), other European cities (Bade, Bijlsma et al. 2017) and in China (Khan, van Nuijs et al. 2014). Its occurrence in wastewater can be only ascribed to illegal disposal or consumption as there is no medical use in Europe (EMCDDA 2011). In this study, a new CF value has been proposed for the first time to allow for the estimation of mephedrone use via WBE. Considering urinary excretion of 15.4%±8.4% as unchanged mephedrone after an oral dose of 150 mg (n=6) (Olesti, Pujadas et al. 2017), CF was set at 6.5. Population-normalised loads ranged throughout a sampling week from 14.9 to 47.7 mg 1000 people^{-1} day^{-1} in the UK (Figures 3 and S2, estimated consumption in Table ~~S16~~S17). Increasing loads were found in weekend days rather than weekdays with a mean value of 25.6 ± 12.0 mg 1000 people^{-1} day^{-1}. A similar trend was observed by Castrignanò et al. (Castrignanò, Lubben et al. 2016), classifying mephedrone as a recreational drug like MDMA. Furthermore, mephedrone was found to be enriched with the *R*-(+)-enantiomer in wastewater ($EF_{ww\ in\ 2014\ (n=6)}$=0.57±0.02 and $EF_{ww\ in\ 2015\ (n=4)}$=0.57±0.04). This indicates that mephedrone was consumed rather than directly disposed (Castrignanò, Mardal et al. 2017~~, Castrignanò E. 2017 (submitted)~~) (see section S4 for further information).

3.4.Other drugs and precursors

The analysis of drug precursors, such as norephedrine, ephedrine and pseudoephedrine (referred in the text as ephedrines), was performed only for Oslo, Bristol, Utrecht (only norephedrine) and Milan (see section S5 for further information).

Mean population-normalised norephedrine loads were 51 mg 1000 people^{-1} day^{-1} in Oslo (probably linked to methamphetamine's metabolism), 7.1 mg 1000 people^{-1} day^{-1} in Milan and 3.4 mg 1000 people^{-1} day^{-1} in Bristol (Table ~~S17~~S18-Figure 4c). Norephedrine was not detected in wastewater from Utrecht. EFs were 0.48±0.04, 0.56±0.11 and 1.00±0.00 (due to <MQL values for the first eluting enantiomer), respectively.

Only two stereoisomers of ephedrine were found in European wastewaters: *1R*,*2S*-(-)-ephedrine and *1S*,*2S*-(+)-pseudoephedrine. Population-normalised *1R*,*2S*-(-)-ephedrine loads were 0.7 mg 1000 people^{-1} day^{-1} in Oslo, 3.4 mg 1000 people^{-1} day^{-1} in Milan and 0.6 mg 1000 people^{-1} day^{-1} in Bristol (Table ~~S18~~S19-Figure 4a). Mean population-normalised *1S*,*2S*-(+)-pseudoephedrine loads were 21.2 mg 1000 people^{-1} day^{-1} in Oslo, 35.7 mg 1000 people^{-1} day^{-1} in Milan and 96.4 mg 1000 people^{-1} day^{-1} in Bristol (Table ~~S18~~S19-Figure 4b).

Chiral PMA (para-methoxyamphetamine), a phenylisopropylamine with hallucinogenic properties, has no legitimate therapeutical use. It is abused alone or in combination with MDMA or PMMA. Seizures have been reported in several European countries, including Belgium, Denmark, Spain, the Netherlands and the UK. However, it was not found in wastewater from any studied city. This is also in accordance with Kinyua et al. (Kinyua, Covaci et al. 2015).

3.5.Consumption estimates of (meth)amphetamine and ephedrines corrected for legal use: a case study in England

In England, legal amphetamine prescriptions in 2015 were as follows: 17.8 kg/year of *S*-(+)-amphetamine (73.4% correction from 23.7 kg/year as dexamfetamine sulphate (Team, Centre et al. 2016) to the free base) and 20.3 kg/year as *S*-(+)-amphetamine (29.7% correction from 68.4 kg/year as lisdexamfetamine dimesylate (Team, Centre et al. 2016) to the free base) (Table 1). Taking into account urinary excretion, the annual amount excreted as *S*-(+)-amphetamine is calculated as 5.2 kg from dexamfetamine sulphate consumption and 8.4 kg from lisdexamfetamine dimesylate. Moreover, 1.3 kg of *R*-(-)-amphetamine was excreted in 2015 from 9.7 kg/year of prescribed selegiline (Team, Centre et al. 2016). As a result, the contribution of legal prescribed and excreted amphetamine to wastewater in the WWTP considered in the study was 1.6 and 0.10 mg day^{-1} 1000 people^{-1} of *S*-(+)- and *R*-(-)-amphetamine, respectively (this does not consider legally purchased drugs traded illegally). Consumption estimates from wastewater analysis were back-calculated by using amphetamine and norephedrine as DTRs (3.3 and 44.7 as corresponding CFs). Despite the good agreement between estimates obtained with considered DTRs, norephedrine is not recommended as a biomarker for amphetamine use as it can result from other sources (e.g. disposal of norephedrine and metabolism of ephedrine and methamphetamine). In relation to these findings, the presence of amphetamine in Bristol was linked to an illegal use of the substance since the contribution of estimates from the legal sources was negligible (Table 1).

Regarding methamphetamine, 2.7 kg/year of the *R*-(-)-enantiomer was excreted into wastewater as a result of 9.7 kg/year of selegiline intake (Team, Centre et al. 2016). Thus, by normalising the data with the population equivalent served by the local WWTP in England, 0.18 mg day^{-1} 1000 people^{-1} of *R*-(-)-methamphetamine (originating from selegiline consumption) was estimated in the studied location. Consumption estimates were performed considering methamphetamine itself, amphetamine and norephedrine as DTRs (see CFs in Table ~~S9~~S10). The estimates obtained with amphetamine and norephedrine as DTR were 100-fold higher than the estimate calculated from methamphetamine. 2.70 mg day^{-1} 1000 people^{-1} of (±)-

methamphetamine, of which 1.8 as *R*-(-)-enantiomer, were estimated by using methamphetamine as DTR, suggesting that its presence was associated mainly with illegal use.

The estimates of the legal use of ephedrines in England in 2015 are as follows (Table 1):

- ephedrine: 0.83 kg/year as hydrochloride (or 0.62 kg/year as free base) resulting in annual excretion of 0.46 kg of ephedrine in England;
- pseudoephedrine: 253.54 kg/year as hydrochloride (or 223.12 kg/year as *1S,2S*-(+)-enantiomer) resulting in annual excretion of 196.34 kg of *1S,2S*-(+)-pseudoephedrine in England;
- norephedrine: 0.35/year and 0.02 kg/year excreted as a result of dexamfetamine sulphate and ephedrine consumption, respectively.

Furthermore, the metabolism of selegiline produces 0.62% (n=4) of (*1S*,*2R*)-(+)-ephedrine, 0.04% (n=4) as (*1R*,*2R*)-(-)-pseudoephedrine and 0.12% (n=4) as (*1S*,*2R*)-(+)-norephedrine (Shin 1997). In 2015 in England, 0.06 kg/year of (*1S*,*2R*)-(+)-ephedrine, 0.004 kg/year of (*1R*,*2R*)-(-)-pseudoephedrine and 0.011 kg/year as (*1S*,*2R*)-(+)-norephedrine were excreted as a result of 9.72 kg/year of selegiline intake (Team, Centre et al. 2016).

Final estimates, normalised with local WWTP, were 0.034, 10.61 and 0.02 mg day^{-1} 1000 people^{-1} of ephedrine, pseudoephedrine and norephedrine respectively (CFs in Table ~~S9~~S10). For Bristol, consumption estimates were in agreement with the legal usage of ephedrine when ephedrine itself was used as DTR and discordant in the case of pseudoephedrine and norephedrine (most likely due to their availability on the OTC market).

4. Conclusions

This study was the first to spatially and temporally assess the enantiomeric profiling of chiral illicit drugs in wastewater serving 4.9 million people in eight European cities. Spatial variations in drug loads were observed across Europe with higher use of amphetamine in Northern European cities, revealing a general enrichment of *R*-(-)-amphetamine in wastewater. The chiral signature of amphetamine revealed that it is present in wastewater as a result of its consumption. High methamphetamine loads were detected in Oslo, where racemic methamphetamine was present, likely due to different trafficking routes from the Baltic countries, rather than Western and Central Europe. The more potent *S*-(+)-methamphetamine was the predominant enantiomer found in wastewater from the other European cities tested, which indicates distribution of enantiopure *S*-(+)-methamphetamine on the illicit market. It could suggest that direct comparison of methamphetamine loads in Oslo and the other European cities should not be undertaken without considering its chiral signature and the different potency of individual enantiomers. The analysis of precursors was compatibly ascribed to their medical use. MDMA was commonly enriched with *R*-(-)-enantiomer in studied European cities, which indicates consumption rather than disposal of the unused drug. MDA was commonly found to be enriched with *S*-(+)-enantiomer, which indicates that its presence in European wastewaters originates from MDMA metabolism (especially during weekends) rather than consumption of MDA itself. However, on a few occasions (UK and The Netherlands), MDA was found to be enriched with *R*-(-)-enantiomer, which indicates its consumption. As MDA is a minor metabolite of MDMA, other metabolites were considered as possible MDMA DTRs, namely HMA and HMMA. HMMA was found to be a suitable MDMA DTR. Furthermore, its chiral signature indicated its enrichment with *S*-(+)-enantiomer, which confirms its origin from MDMA metabolism. Population-normalised mephedrone loads were up to 47.7 mg 1000 people^{-1} day^{-1} in wastewater in the UK, where an enrichment of *R*-(+)-enantiomer suggested stereoselective metabolism in humans, indicating consumption rather than direct disposal.

Contributions

This work was supported by the European Union's Seventh Framework Programme for Research, Technological Development and Demonstration [grant agreement 317205, the SEWPROF MC ITN project, 'A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level']. Wastewater samples were provided by local WWTPs to the University of Bath (United Kingdom) by: Wessex Water, Norwegian Institute for Water Research (Norway), Swiss Federal Institute of Aquatic Science and Technology (Switzerland), Technical University of Denmark (Denmark), Mario Negri Institute for Pharmacological Research (Italy), University of Antwerp (Belgium), KWR Watercycle Research Institute (The Netherlands), University Jaume I (Spain). Erika Castrignanò and Barbara Kasprzyk-Hordern planned and designed the study. Erika Castrignanò, Zhugen Yang, Richard Bade, J. Baz-Lomba, Sara Castiglioni, Ana Causanilles, Adrian Covaci, Emma Gracia-Lor, Felix Hernandez, Juliet Kinyua, Ann-Kathrin McCall, Alexander L. N. van Nuijs, Christoph Ort, Benedek G. Plósz, Pedram Ramin, Nikolaos I. Rousis, Yeonsuk Ryu, Kevin V Thomas, Pim de Voogt, Ettore Zuccato and Barbara Kasprzyk-Hordern organised the collection of the wastewater samples. Erika Castrignanò prepared and analysed the samples, interpreted the results. Erika Castrignanò and Barbara Kasprzyk-Hordern drafted the manuscript, which was critically revised by all co-authors.

References

Bade, R., L. Bijlsma, J. V. Sancho, J. A. Baz-Lomba, S. Castiglioni, E. Castrignanò, A. Causanilles, E. Gracia-Lor, B. Kasprzyk-Hordern and J. Kinyua (2017). "Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities." Chemosphere **168**: 1032-1041.

Baselt, R. C. Disposition of Toxic Drugs and Chemicals in Man. Chemical Toxicology Institute, Foster City, CA.

Baz-Lomba, J. A., S. Salvatore, E. Gracia-Lor, R. Bade, S. Castiglioni, E. Castrignano, A. Causanilles, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, A. van Nuijs, C. Ort, B. G. Plosz, P. Ramin, M. Reid, N. I. Rousis, Y. Ryu, P. de Voogt, J. Bramness and K. Thomas (2016). "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities." BMC Public Health **16**(1): 1035.

Camacho-Muñoz, D. (2015). "Enantiomeric Profiling of Chiral Pharmacologically Active Compounds in the Environment with the usage of chiral Liquid Chromatography Coupled with Tandem Mass Spectrometry." Current Analytical Chemistry **12**.

Castiglioni, S., A. Borsotti, I. Senta and E. Zuccato (2015). "Wastewater analysis to monitor spatial and temporal patterns of use of two synthetic recreational drugs, ketamine and mephedrone, in Italy." Environ Sci Technol **49**(9): 5563-5570.

Castiglioni, S., I. Senta, A. Borsotti, E. Davoli and E. Zuccato (2014). "A novel approach for monitoring tobacco use in local communities by wastewater analysis." Tob Control.

Castiglioni, S., K. V. Thomas, B. Kasprzyk-Hordern, L. Vandam and P. Griffiths (2014). "Testing wastewater to detect illicit drugs: state of the art, potential and research needs." Sci Total Environ **487**: 613-620.

Castrignanò, E., A. Lubben and B. Kasprzyk-Hordern (2016). "Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry." J Chromatogr A **1438**: 84-99.

Castrignanò, E., M. Mardal, A. Rydevik, B. Miserez, J. Ramsey, T. Shine, G. D. Pantoș, M. R. Meyer and B. Kasprzyk-Hordern (2017). "A new approach towards biomarker selection in estimation of human exposure to chiral chemicals: a case study of mephedrone." Scientific Reports.

~~Castrignanò E., M. M., Rydevik A., Miserez B., Ramsey J., Shine T., Pantoș G.D., Meyer M.R., Kasprzyk-Hordern B. (2017 (submitted)). "A new approach towards biomarker selection in estimation of human exposure to chiral chemicals: a case study of mephedrone." Scientific Reports.~~

Causanilles, A., E. Emke and P. de Voogt (2016). "Determination of phosphodiesterase type V inhibitors in wastewater by direct injection followed by liquid chromatography coupled to tandem mass spectrometry." Sci Total Environ **565**: 140-147.

Chiaia-Hernandez, A. C., C. J. Banta-Green and J. A. Field (2011). "Interpreting methamphetamine levels in a high-use community." Environmental Science and Pollution Research **18**(9): 1471-1477.

EMCDDA (2011). Report on the risk assessment of mephedrone in the framework of the Council Decision on new psychoactive substances. Lisbon, EMCDDA**:** 200.

EMCDDA (2014). "Exploring methamphetamine trends in Europe." 10.

EMCDDA (2015). "European Drug Report 2015: Trends and Developments."

Emke, E., S. Evans, B. Kasprzyk-Hordern and P. de Voogt (2014). "Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: a Dutch perspective." Sci Total Environ **487**: 666-672.

Freeman, S. and J. F. Alder (2002). "Arylethylamine psychotropic recreational drugs: a chemical perspective." European journal of medicinal chemistry **37**(7): 527-539.

González-Mariño, I., E. Gracia-Lor, N. I. Rousis, E. Castrignanò, K. V. Thomas, J. B. Quintana, B. Kasprzyk-Hordern, E. Zuccato and S. Castiglioni (2016). "Wastewater-based epidemiology to monitor synthetic cathinones use in different European countries." Environmental Science & Technology **50**(18): 10089-10096.

Gonzalez-Marino, I., E. Zuccato, M. M. Santos and S. Castiglioni (2017). "Monitoring MDMA metabolites in urban wastewater as novel biomarkers of consumption." Water Res **115**: 1-8.

http://www.emcdda.europa.eu/activities/wastewater-analysis. (2017). from http://www.webcitation.org/6ugBHKLDN.

http://www.emcdda.europa.eu/topics/pods/waste-water-analysis. (2016, June 2017). "Wastewater analysis and drugs — a European multi-city study " Retrieved January, 2016, from http://www.webcitation.org/6uhGh4vmB.

Karch, S. B. and O. Drummer (2001). Karch's pathology of drug abuse, CRC press.

Karolak, S., T. Nefau, E. Bailly, A. Solgadi and Y. Levi (2010). "Estimation of illicit drugs consumption by wastewater analysis in Paris area (France)." Forensic Science International **200**(1–3): 153-160.

Kasprzyk-Hordern, B. (2010). "Pharmacologically active compounds in the environment and their chirality." Chemical Society Reviews **39**(11): 4466-4503.

Kasprzyk-Hordern, B. and D. R. Baker (2012). "Estimation of community-wide drugs use via stereoselective profiling of sewage." Science of the Total Environment **423**: 142-150.

Kasprzyk-Hordern, B., L. Bijlsma, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, F. Hernandez, C. Ort, M. Reid and A. van Nuijs (2014). "Wastewater-based epidemiology for public health monitoring." Water and Sewerage Journal **4**: 25.

Kasprzyk-Hordern, B., R. M. Dinsdale and A. J. Guwy (2009). "Illicit drugs and pharmaceuticals in the environment--forensic applications of environmental data. Part 1: Estimation of the usage of drugs in local communities." Environ Pollut **157**(6): 1773-1777.

Kasprzyk-Hordern, B., V. V. Kondakal and D. R. Baker (2010). "Enantiomeric analysis of drugs of abuse in wastewater by chiral liquid chromatography coupled with tandem mass spectrometry." Journal of Chromatography A **1217**(27): 4575-4586.

Khan, U., A. L. van Nuijs, J. Li, W. Maho, P. Du, K. Li, L. Hou, J. Zhang, X. Meng, X. Li and A. Covaci (2014). "Application of a sewage-based approach to assess the use of ten illicit drugs in four Chinese megacities." Sci Total Environ **487**: 710-721.

Kinyua, J., A. Covaci, W. Maho, A. K. McCall, H. Neels and A. L. van Nuijs (2015). "Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS." Drug Test Anal **7**(9): 812-818.

Lai, F. Y., J. W. O'Brien, P. K. Thai, W. Hall, G. Chan, R. Bruno, C. Ort, J. Prichard, S. Carter and S. Anuj (2016). "Cocaine, MDMA and methamphetamine residues in wastewater: Consumption trends (2009–2015) in South East Queensland, Australia." Science of the Total Environment **568**: 803-809.

Levine, B. (2003). Principles of forensic toxicology, Amer. Assoc. for Clinical Chemistry.

McCall, A.-K., A. Scheidegger, M. M. Madry, A. E. Steuer, D. G. Weissbrodt, P. A. Vanrolleghem, T. Kraemer, E. Morgenroth and C. Ort (2016). "Influence of Different Sewer Biofilms on Transformation Rates of Drugs." Environmental Science & Technology **50**(24): 13351-13360.

Metcalfe, C., K. Tindale, H. Li, A. Rodayan and V. Yargeau (2010). "Illicit drugs in Canadian municipal wastewater and estimates of community drug use." Environ Pollut **158**(10): 3179-3185.

Meyer, M. R., F. T. Peters and H. H. Maurer (2009). "Investigations on the human hepatic cytochrome P450 isozymes involved in the metabolism of 3, 4-methylenedioxy-amphetamine (MDA) and benzodioxolyl-butanamine (BDB) enantiomers." Toxicology letters **190**(1): 54-60.

Nefau, T., S. Karolak, L. Castillo, V. Boireau and Y. Levi (2013). "Presence of illicit drugs and metabolites in influents and effluents of 25 sewage water treatment plants and map of drug consumption in France." Sci Total Environ **461-462**: 712-722.

Olesti, E., M. Pujadas, E. Papaseit, C. Pérez-Mañá, Ó. J. Pozo, M. Farré and R. de la Torre (2017). "GC–MS Quantification Method for Mephedrone in Plasma and Urine: Application to Human Pharmacokinetics." Journal of Analytical Toxicology **41**(2): 100-106.

Ort, C., A. L. van Nuijs, J. D. Berset, L. Bijlsma, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, D. Fatta-Kassinos, P. Griffiths, F. Hernandez, I. Gonzalez-Marino, R. Grabic, B. Kasprzyk-Hordern, N. Mastroianni, A. Meierjohann, T. Nefau, M. Ostman, Y. Pico, I. Racamonde, M. Reid, J. Slobodnik, S. Terzic, N. Thomaidis and K. V. Thomas (2014). "Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis." Addiction **109**(8): 1338-1352.

Petrie, B., J. Youdan, R. Barden and B. Kasprzyk-Hordern (2016). "New Framework To Diagnose the Direct Disposal of Prescribed Drugs in Wastewater - A Case Study of the Antidepressant Fluoxetine." Environ Sci Technol.

Postigo, C., M. J. Lopez de Alda and D. Barcelo (2010). "Drugs of abuse and their metabolites in the Ebro River basin: occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation." Environ Int **36**(1): 75-84.

Ramin, P., A. Libonati Brock, F. Polesel, A. Causanilles, E. Emke, P. de Voogt and B. G. Plósz (2016). "Transformation and Sorption of Illicit Drug Biomarkers in Sewer Systems: Understanding the Role of Suspended Solids in Raw Wastewater." Environmental Science & Technology **50**(24): 13397-13408.

Reid, M. J., L. Derry and K. V. Thomas (2014). "Analysis of new classes of recreational drugs in sewage: synthetic cannabinoids and amphetamine-like substances." Drug Test Anal **6**(1-2): 72-79.

Reid, M. J., K. H. Langford, J. Morland and K. V. Thomas (2011). "Quantitative assessment of time dependent drug-use trends by the analysis of drugs and related metabolites in raw sewage." Drug Alcohol Depend **119**(3): 179-186.

Reid, M. J., K. H. Langford, J. Mørland and K. V. Thomas (2011). "Analysis and interpretation of specific ethanol metabolites, ethyl sulfate, and ethyl glucuronide in sewage effluent for the quantitative measurement of regional alcohol consumption." Alcoholism: Clinical and Experimental Research **35**(9): 1593-1599.

Schwaninger, A. E., M. R. Meyer, A. J. Barnes, E. A. Kolbrich-Spargo, D. A. Gorelick, R. S. Goodwin, M. A. Huestis and H. H. Maurer (2012). "Stereoselective urinary MDMA (ecstasy) and metabolites excretion kinetics following controlled MDMA administration to humans." Biochemical pharmacology **83**(1): 131-138.

Shin, H.-S. (1997). "Metabolism of Selegiline in Humans." Identification, Excretion, and Stereochemistry of Urine Metabolites **25**(6): 657-662.

Team, P. M., H. a. S. C. I. Centre and P. o. t. G. S. Service (2016). Prescription Cost Analysis: England 2015**:** 711.

Terzic, S., I. Senta and M. Ahel (2010). "Illicit drugs in wastewater of the city of Zagreb (Croatia)--estimation of drug abuse in a transition country." Environ Pollut **158**(8): 2686-2693.

Thomas, K. V., L. Bijlsma, S. Castiglioni, A. Covaci, E. Emke, R. Grabic, F. Hernandez, S. Karolak, B. Kasprzyk-Hordern, R. H. Lindberg, M. Lopez de Alda, A. Meierjohann, C. Ort, Y. Pico, J. B. Quintana, M. Reid, J. Rieckermann, S. Terzic, A. L. van Nuijs and P. de Voogt (2012). "Comparing illicit drug use in 19 European cities through sewage analysis." Sci Total Environ **432**: 432-439.

Thomas, K. V. and M. J. Reid (2011). "What Else Can the Analysis of Sewage for Urinary Biomarkers Reveal About Communities?" Environmental Science & Technology **45**(18): 7611-7612.

Tscharke, B. J., C. Chen, J. P. Gerber and J. M. White (2016). "Temporal trends in drug use in Adelaide, South Australia by wastewater analysis." Science of The Total Environment **565**: 384-391.

van Nuijs, A. L., J. F. Mougel, I. Tarcomnicu, L. Bervoets, R. Blust, P. G. Jorens, H. Neels and A. Covaci (2011). "Sewage epidemiology--a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium." Environ Int **37**(3): 612-621.

van Nuijs, A. L. N., S. Castiglioni, I. Tarcomnicu, C. Postigo, M. L. de Alda, H. Neels, E. Zuccato, D. Barcelo and A. Covaci (2011). "Illicit drug consumption estimations derived from wastewater analysis: A critical review." Science of the Total Environment **409**(19): 3564-3577.

Van Nuijs, A. L. N., B. Pecceu, L. Theunis, N. Dubois, C. Charlier, P. G. Jorens, L. Bervoets, R. Blust, H. Meulemans, H. Neels and A. Covaci (2009). "Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium." Addiction **104**(5): 734-741.

van Nuijs, A. L. N., B. Pecceu, L. Theunis, N. Dubois, C. Charlier, P. G. Jorens, L. Bervoets, R. Blust, H. Neels and A. Covaci (2009). "Spatial and temporal variations in the occurrence of cocaine and benzoylecgonine in waste- and surface water from Belgium and removal during wastewater treatment." Water Research **43**(5): 1341-1349.

Vazquez-Roig, P., B. Kasprzyk-Hordern, C. Blasco and Y. Picó (2014). "Stereoisomeric profiling of drugs of abuse and pharmaceuticals in wastewaters of Valencia (Spain)." Science of The Total Environment **494–495**(0): 49-57.

Zuccato, E., C. Chiabrando, S. Castiglioni, R. Bagnati and R. Fanelli (2008). "Estimating community drug abuse by wastewater analysis." Environmental Health Perspectives **116**(8): 1027-1032.

Zuccato, E., C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea and R. Fanelli (2005). "Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse." Environ Health **4**: 14.